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Use of Epidemiological Cutoff Values To Examine 9-Year Trends in Susceptibility of *Aspergillus* Species to the Triazoles[▽]

M. Pfaller,* L. Boyken, R. Hollis, J. Kroeger, S. Messer, S. Tendolkar, and D. Diekema

University of Iowa, Iowa City, Iowa

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In the absence of clinical breakpoints, epidemiological cutoff values (ECVs) have been established to distinguish wild-type (WT) isolates of *Aspergillus* spp. from those that may harbor resistance mutations. Recently, the CLSI has developed ECVs for triazoles (itraconazole, posaconazole, and voriconazole) and common *Aspergillus* species. We applied the triazole ECVs to 1,789 *Aspergillus* isolates collected from 63 centers worldwide from 2001 to 2009 to determine the frequency of non-WT strains of each species. Temporal trends were evaluated for *Aspergillus fumigatus* and *Aspergillus flavus* over the 9-year period for each drug. The collection included 1,312 isolates of *A. fumigatus*, 235 of *A. flavus*, 162 of *Aspergillus niger*, 64 of *Aspergillus terreus*, and 15 of *Aspergillus versicolor*. Using the ECVs, the percentages of non-WT isolates for itraconazole, posaconazole, and voriconazole, respectively, were as follows: *A. fumigatus* (2.0%, 3.5%, and 1.4%), *A. flavus* (0.8%, 5.1%, and 1.7%), *A. niger* (17.3%, 3.7%, and 0.6%), *A. terreus* (0.0%, 1.6%, and 3.2%), and *A. versicolor* (6.3%, 0.0%, and 0.0%). Among 49 *Aspergillus* isolates for which itraconazole MICs were >2 μg/ml, the posaconazole and voriconazole MICs were greater than the ECVs for 14 and 12 isolates, respectively. The percentages of isolates for which MICs were greater than the ECVs ranged from 1.1 to 5.7% for posaconazole, 0.0 to 1.6% for voriconazole, and 0.7 to 4.0% for itraconazole. There was no consistent trend toward decreased susceptibility for any triazole and *A. fumigatus* or *A. flavus* over time. Decreased susceptibility among *Aspergillus* spp. was observed for each of the extended-spectrum triazoles and varied by species over the 9-year study period.

The extended-spectrum triazoles, itraconazole, posaconazole, and voriconazole, are important antifungal agents in the prevention and treatment of invasive aspergillosis (IA) (32). Although resistance to these agents is considered to be rare, increased resistance has been noted in several geographic regions since 1999 (1, 3, 10–12, 19, 25, 28–31). Recent evidence from Denmark and the Netherlands suggests the possibility that azole resistance in *Aspergillus fumigatus* may be a side effect of environmental fungicide use (19, 31). The mechanisms of resistance to the triazoles are best studied in *A. fumigatus* and involve mutations in the *CYP51A* gene encoding the target enzyme as well as efflux and the overexpression of *CYP51A* (1–5, 10–12, 15–20, 23–25). Specific mutations in *CYP51A* may result in resistance to one, two, or all three triazoles (11, 18, 23, 30). These observations indicate that triazole resistance among *Aspergillus* spp. may be more common than currently acknowledged and that clinical microbiology laboratories should determine the *in vitro* susceptibility of clinically relevant isolates of *Aspergillus* spp. (1, 3, 8, 12, 14, 23, 30). In the absence of clinical breakpoints (CBPs), epidemiological cutoff values (ECVs) have been established as a means of distinguishing wild-type (WT) isolates of *Aspergillus* spp. from those that may exhibit acquired azole resistance mechanisms (8, 22, 23, 30). Recently, the Clinical and Laboratory Standards Institute (CLSI) has developed ECVs for all three triazoles and the six most common species of *Aspergillus* (8) (Table 1). In this study, we have applied these ECVs to a global collection of 1,789 clinical respiratory tract isolates of *Aspergillus* spp. collected from more than 60 medical centers from 2001 through 2009 to determine the frequency of non-WT strains of each species over time.

MATERIALS AND METHODS

Organisms. A total of 1,789 clinical isolates of *Aspergillus* spp. obtained from more than 60 medical centers worldwide from 2001 through 2009 were tested against itraconazole, posaconazole, and voriconazole. The collection included 1,312 isolates of *A. fumigatus*, 235 of *Aspergillus flavus*, 162 of *Aspergillus niger*, 64 of *Aspergillus terreus*, and 16 of *Aspergillus versicolor*. The isolates were obtained from a variety of sources, including sputum, bronchoscopy, and tissue biopsy specimens and represented individual infectious episodes. The isolates were collected at individual study sites and were sent to the University of Iowa (Iowa City) for identification and susceptibility testing as described previously (22). All isolates were identified by standard microscopic morphology and were stored as spore suspensions in sterile distilled water at room temperature until used in the study. Before testing, each isolate was subcultured at least twice on potato dextrose agar (Remel, Lenexa, KS) to ensure viability and purity. As a screen for cryptic species within the *A. fumigatus* complex (e.g., *Aspergillus lentulus*), all *A. fumigatus* isolates were tested for growth at 50°C. All isolates screened grew at 50°C, confirming that they were likely to be *A. fumigatus*. A subset of 499 isolates of *A. fumigatus* were investigated further using molecular methods, and all were confirmed to be *A. fumigatus* (9).

Antifungal susceptibility testing. Itraconazole, posaconazole, and voriconazole were all obtained as reagent-grade powders from their respective manufacturers. The CLSI broth microdilution (BMD) method was performed according to the M38-A2 standard (6) by using RPMI 1640 medium, an inoculum of 0.4×10^4 to 5×10^4 CFU/ml, and incubation at 35°C. The MIC was determined visually after 48 h of incubation as the lowest concentration of drug that produced complete inhibition of growth.

Quality control. Quality control was ensured by testing the following strains recommended in CLSI document M38-A2 (6): *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, and *A. flavus* ATCC 204304.

* Corresponding author. Mailing address: Medical Microbiology Division, C606 GH, Department of Pathology, University of Iowa College of Medicine, Iowa City, IA 52242. Phone: (319) 356-8615. Fax: (319) 356-4916. E-mail: michael-pfaller@uiowa.edu.

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TABLE 1. Epidemiological cutoff values (ECVs) for itraconazole, posaconazole, and voriconazole and five species of *Aspergillus*[a]

Species	Antifungal agent	No. tested	MIC (μg/ml)			% of isolates at or below the ECV
			Range	Mode	ECV	
A. fumigatus	Itraconazole	2,591	≤0.03–≥4	0.5	1	98.8
	Posaconazole	1,684	≤0.015–≥4	0.06	0.5	97.8
	Voriconazole	2,815	0.03–≥4	0.25	1	98.2
A. flavus	Itraconazole	538	0.03–≥4	0.5	1	99.6
	Posaconazole	444	≤0.03–>4	0.06	0.5	99.1
	Voriconazole	592	0.06–≥4	0.5	1	98.3
A. terreus	Itraconazole	369	0.03–1	0.25	1	100.0
	Posaconazole	330	≤0.03–2	0.25	0.5	99.7
	Voriconazole	462	0.03–≥4	0.5	1	99.1
A. niger	Itraconazole	468	0.03–≥4	1	2	100.0
	Posaconazole	366	≤0.03–2	0.5	1	98.2
	Voriconazole	520	≤0.03–≥4	1	2	99.2
A. versicolor	Itraconazole	59	0.03–≥4	1	2	100.0
	Posaconazole	32	0.03–≥4	0.5	4	98.4
	Voriconazole	71	0.03–≥4	0.25	2	97.5
A. nidulans	Itraconazole	143	0.03–≥4	0.5	1	95.0
	Posaconazole	131	0.03–2	0.25	1	98.4
	Voriconazole	141	0.03–≥4	0.12	2	98.6

[a] Data compiled from Espinel-Ingroff et al. (8).

Definitions. The definitions of WT and ECVs were outlined previously (8, 13, 22, 23, 27). A WT organism is defined as a strain which does not harbor any acquired resistance to the particular antimicrobial agent being examined (8, 22, 27). The typical MIC distribution for WT organisms covers three to five 2-fold dilution steps surrounding the modal MIC (8, 22, 23).

The ECV for each triazole and species of *Aspergillus* was obtained as described previously (8, 22) by considering the WT MIC distribution, the modal MIC for each distribution, and the inherent variability of the test. In general, the ECV should encompass at least 95% of isolates in the WT distribution (26, 27) (Table 1). Organisms with acquired resistance mechanisms may be identified as those with a MIC higher than the highest MIC of the WT (greater than the ECV) (23). The ECV can be used as the most sensitive measure of the emergence of strains with reduced susceptibility to a given agent (13, 22, 23, 30).

RESULTS AND DISCUSSION

The ECVs for each triazole and the six species of *Aspergillus* are shown in Table 1. The ECVs were determined in an earlier multicenter study of more than 4,000 isolates of *Aspergillus* spp. tested against all three triazoles using the CLSI BMD method (8). Previous studies have demonstrated the ability of these triazole ECVs to discriminate WT strains (MIC values less than or equal to the ECV) from those with acquired resistance mechanisms (11, 23, 30).

Although ECVs may be considered to be an early step in the development of CBPs (13, 30), the most important role for these cutoffs is to detect the emergence of reduced susceptibility to the agent of interest in the context of a resistance surveillance program. This is especially important in the case of the triazoles and *Aspergillus* spp. due to the lack of CBPs, the important role of these agents in the treatment of IA, and the fact that clinically important resistance to these agents appears to be increasing in some geographic regions (3, 11, 12, 19, 30).

In Table 2, we have applied the ECVs derived by Espinel-Ingroff et al. (8) to our collection of *Aspergillus* isolates spanning a 9-year period from 2001 to 2009. Application of the ECVs shows that the frequency of non-WT isolates of each species ranged from 1.4% (voriconazole) to 3.5% (posaconazole) for *A. fumigatus*, from 0.8% (itraconazole) to 5.1% (posaconazole) for *A. flavus*, from 0.6% (voriconazole) to 17.3% (itraconazole) for *A. niger*, from 0.0% (itraconazole) to 3.2% (voriconazole) for *A. terreus*, and from 0.0% (posaconazole and voriconazole) to 6.3% (itraconazole) for *A. versicolor*. Little comparable data are available from other surveys with the exception of *A. fumigatus* in the United Kingdom and the Netherlands (3, 11, 30). Howard et al. (11) found that 5% of 400 isolates of *A. fumigatus* tested at a reference laboratory in Manchester, United Kingdom, were resistant to itraconazole, and Verweij et al. (30) found azole resistance in 5% to 10% of isolates from the Netherlands over the time period of 1998 to 2007.

One aspect of the data shown in Table 2 that differs from previous surveys (3, 11, 12, 30) is the finding of more isolates of *A. fumigatus* that appear less susceptible (MIC > ECV) to posaconazole than to itraconazole. This cannot be accounted for by any of the known mechanisms of resistance described to date for *A. fumigatus* (1–5, 7, 12, 18, 23, 30) and could possibly be caused by laboratory-based issues, such as the poor solubility characteristics of posaconazole, which could lead to a greater variability in the MIC distribution. We have examined this issue extensively and have retested the non-WT strains of *A. fumigatus* from 2008 and 2009 both within our own laboratory (at least three times each, including with a new batch of posaconazole) and in an outside laboratory and have confirmed the high MIC values. Furthermore, we have not detected any shift or drift in the posaconazole or itraconazole quality control values that suggest excessive methodological variation. It should be noted that Verweij et al. (30) reported a substantial number of *A. fumigatus* isolates for which the

TABLE 2. Frequency of non-WT strains of *Aspergillus* species determined by CLSI BMD testing of itraconazole, posaconazole, and voriconazole

Species	Antifungal agent	ECV (μg/ml)	No. tested	MIC (μg/ml)		% of MICs >ECV
				Range	Mode[a]	
A. fumigatus	Itraconazole	1	1,221	0.015–>8	0.25	2.0
	Posaconazole	0.5	1,312	0.007–2	0.03	3.5
	Voriconazole	1	1,312	0.06–4	0.25	1.4
A. flavus	Itraconazole	1	225	0.06–>8	0.12	0.8
	Posaconazole	0.5	235	0.015–>8	0.06	5.1
	Voriconazole	1	235	0.12–>8	0.5	1.7
A. niger	Itraconazole	2	155	0.003–>8	1	17.3
	Posaconazole	1	162	0.007–2	0.12	3.7
	Voriconazole	2	161	0.12–4	1	0.6
A. terreus	Itraconazole	1	54	0.03–1	NM	0.0
	Posaconazole	0.5	64	0.015–2	0.06	1.6
	Voriconazole	1	64	0.25–>8	0.5	3.2
A. versicolor	Itraconazole	2	16	0.06–>8	0.25	6.3
	Posaconazole	4	16	0.03–1	NM	0.0
	Voriconazole	2	16	0.03–2	0.25	0.0

[a] NM, no mode.

posaconazole MICs fell just beyond (1 dilution) the EUCAST ECV, which they categorized as "intermediate" in susceptibility to this agent. This designation may be appropriate for similar strains in our survey. These findings may suggest that the ECV for posaconazole and *A. fumigatus* is too low. Alternatively, they could represent an as-yet-uncharacterized acquired resistance mechanism. Studies to address these issues are under way.

Temporal trends in decreased susceptibility were evaluated for *A. fumigatus* and *A. flavus* over the 9-year period for each triazole (Table 3). The proportion of isolates of *A. fumigatus* that were non-WT ranged from 0.7% to 4.0% for itraconazole, from 1.1% to 5.7% for posaconazole, and from 0.0% to 1.6% for voriconazole. Likewise the proportion of *A. flavus* isolates that were non-WT ranged from 0.0% to 3.0% for itraconazole, from 1.3% to 9.4% for posaconazole, and from 0.0% to 2.6% for voriconazole. In contrast to the data from the United Kingdom and the Netherlands, we found no consistent trend toward decreased susceptibility for any triazole and *A. fumigatus* over time (8, 25).

It should be noted that among the non-WT isolates of *A. fumigatus*, the geographic origins were quite diverse, with isolates originating from centers in Australia, China, Europe, South America, and the United States. In 2008, we did observe

TABLE 3. Trends in susceptibility of *A. fumigatus* and *A. flavus* respiratory tract isolates to itraconazole, posaconazole, and voriconazole as determined by CLSI BMD methods

Antifungal agent	Species (ECV [μg/ml])	Years	No. tested	MIC (μg/ml)		% of MICs >ECV
				Range	Mode	
Itraconazole	*A. fumigatus* (1)	2001–2003	173	0.12–2	0.5	4.0
	A. fumigatus (1)	2004–2006	441	0.03–>8	0.25	0.7
	A. fumigatus (1)	2007–2009	607	0.015–>8	0.25	2.3
	A. flavus (1)	2001–2003	32	0.25–1	0.5	0.0
	A. flavus (1)	2004–2006	68	0.06–>8	0.12	3.0
	A. flavus (1)	2007–2009	125	0.06–1	0.12	0.0
Posaconazole	*A. fumigatus* (0.5)	2001–2003	173	0.015–2	0.25	5.7
	A. fumigatus (0.5)	2004–2006	532	0.007–1	0.03	1.1
	A. fumigatus (0.5)	2007–2009	607	0.015–2	0.03	4.9
	A. flavus (0.5)	2001–2003	32	0.12–2	0.25	9.4
	A. flavus (0.5)	2004–2006	78	0.015–>8	0.06	1.3
	A. flavus (0.5)	2007–2009	125	0.03–1	0.06	6.4
Voriconazole	*A. fumigatus* (1)	2001–2003	173	0.06–1	0.25	0.0
	A. fumigatus (1)	2004–2006	532	0.12–4	0.25	1.6
	A. fumigatus (1)	2007–2009	607	0.12–4	0.5	1.6
	A. flavus (1)	2001–2003	32	0.12–1	0.5	0.0
	A. flavus (1)	2004–2006	78	0.25–>8	0.5	2.6
	A. flavus (1)	2007–2009	125	0.25–2	1	1.6

TABLE 4. Cross-resistance between itraconazole, posaconazole, and voriconazole among 49 isolates of *Aspergillus* spp. with decreased susceptibility to itraconazole[a]

Aspergillus species (no. tested)	Antifungal agent	ECV (μg/ml)	MIC (μg/ml)				% of MICs ≤ECV
			Range	Mode	50%	90%	
A. flavus (2)	Itraconazole	1	4–>8	NM	4	NC	0.0
	Posaconazole	0.5	0.12–>8	NM	0.12	NC	50.0
	Voriconazole	1	1–>8	NM	1	NC	50.0
A. fumigatus (13)	Itraconazole	1	>8	>8	>8	>8	0.0
	Posaconazole	0.5	0.25–2	1	1	2	30.8
	Voriconazole	1	0.25–4	NM	2	4	42.6
A. niger (27)	Itraconazole	2	4–>8	>8	>8	>8	0.0
	Posaconazole	1	0.06–1	0.25	0.25	0.5	100.0
	Voriconazole	2	0.5–2	1	1	2	100.0
A. glaucus (4)	Itraconazole	NA	4–>8	>8	>8	>8	NA
	Posaconazole	NA	0.25–>8	>8	>8	>8	NA
	Voriconazole	NA	1–8	8	4	8	NA
A. nidulans (1)	Itraconazole	1	>8	NC	NC	NC	0.0
	Posaconazole	1	>8	NC	NC	NC	0.0
	Voriconazole	2	>8	NC	NC	NC	0.0
A. versicolor (1)	Itraconazole	2	>8	NC	NC	NC	0.0
	Posaconazole	4	1	NC	NC	NC	100.0
	Voriconazole	2	0.25	NC	NC	NC	100.0
A. sydowii (1)	Itraconazole	NA	4	NC	NC	NC	NA
	Posaconazole	NA	1	NC	NC	NC	NA
	Voriconazole	NA	2	NC	NC	NC	NA

[a] Itraconazole MICs for all isolates were ≥4 μg/ml. NM, no mode; NC, value not calculated; NA, data not available.

several non-WT strains originating from different centers in Hangzhou, China, but further characterization revealed these strains to be nonclonal in nature (data not shown).

There were 49 isolates in the collection for which itraconazole MICs were ≥4 μg/ml, 44 of which were species for which ECVs have been determined for itraconazole, posaconazole, and voriconazole (Table 4). Among the 44 isolates for which ECVs have been determined, the posaconazole and voriconazole MICs were greater than the ECVs for 11 (25%) and 9 (20%) isolates, respectively. The slightly greater cross-resistance between itraconazole and posaconazole is similar to that reported elsewhere (5, 8, 16, 19, 21, 23, 25). Notably, cross-resistance was not detected among the 27 itraconazole-resistant isolates of *A. niger*. Although ECVs have not been determined for the azoles and *Aspergillus glaucus*, three of the four itraconazole-resistant strains showed decreased susceptibility to both posaconazole and voriconazole (MICs, ≥4 μg/ml).

In summary, we have demonstrated the utility of ECVs in detecting the emergence of decreased susceptibility to the triazoles in surveys of antifungal resistance in *Aspergillus* spp. Decreased susceptibility among *Aspergillus* spp. was observed for each of the extended-spectrum triazoles and varied according to species over the 9-year study period. Cross-resistance to posaconazole and voriconazole was detected among itraconazole-resistant strains of *A. flavus*, *A. fumigatus*, *A. glaucus*, and *A. nidulans*. Application of *in vitro* susceptibility testing and the use of ECVs are important in determining resistance trends among the triazoles and *Aspergillus* spp.

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REFERENCES

1. **Arendrup, M. C., et al.** 2010. Development of azole resistance in *Aspergillus fumigatus* during azole therapy associated with change in virulence. PLoS One **5:**e10080.
2. **Balashov, S. V., R. Gardiner, S. Park, and D. S. Perlin.** 2005. Rapid, high-throughput, multiplex, real-time PCR for identification of mutations in the *cyp51A* gene of *Aspergillus fumigatus* that confer resistance to itraconazole. J. Clin. Microbiol. **43:**214–222.
3. **Bueid, A., et al.** 2010. Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. J. Antimicrob. Chemother. **65:**2116–2118.
4. **Cannon, R. D., et al.** 2009. Efflux-mediated antifungal resistance. Clin. Microbiol. Rev. **22:**291–321.
5. **Chen, J., H. Li, R. Li, D. Bu, and Z. Wan.** 2005. Mutations in the *cyp51A* gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. J. Antimicrob. Chemother. **55:**31–37.
6. **CLSI.** 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi, 2nd ed. Approved standard M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
7. **Diaz-Guerra, T. M., E. Mellado, M. Cuenca-Estrella, and J. L. Rodriguez-Tudela.** 2003. A point mutation in the 14-α-sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. Antimicrob. Agents Chemother. **47:**1120–1124.
8. **Espinel-Ingroff, A., et al.** 2010. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). J. Clin. Microbiol. **48:** 3251–3257.
9. **Etienne, K. A., et al.** 2009. Screening of a large global *Aspergillus fumigatus* species complex collection by using a species-specific microsphere-based Luminex assay. J. Clin. Microbiol. **47:**4171–4172.
10. **Howard, S. J., et al.** 2006. Multi-azole resistance in *Aspergillus fumigatus*. Int. J. Antimicrob. Agents **28:**450–453.

11. **Howard, S. J., et al.** 2009. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. Emerg. Infect. Dis. **15:**1068–1076.
12. **Howard, S. J., and M. C. Arendrup.** 2010. Acquired antifungal resistance in *Aspergillus fumigatus*: epidemiology and detection. Med. Mycol. [Epub ahead of print.] doi:10.3109/13693786.2010.508469.
13. **Kahlmeter, G., et al.** 2003. European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. J. Antimicrob. Chemother. **52:**145–148.
14. **Lass-Florl, C.** 2009. Azole resistance in aspergillosis: the next threat? Curr. Fungal Infect. Rep. **3:**236–242.
15. **Mann, P. A., et al.** 2003. Mutations in *Aspergillus fumigatus* resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14α-demethylase. Antimicrob. Agents Chemother. **47:**577–581.
16. **Mellado, E., G. Garcia-Effron, L. Alcazar-Fuoli, M. Cuenca-Estrella, and J. L. Rodriguez-Tudela.** 2004. Substitutions at methionine 220 in the 14α-sterol demethylase (Cyp51A) of *Aspergillus fumigatus* are responsible for resistance in vitro to azole antifungal drugs. Antimicrob. Agents Chemother. **48:**2747–2750.
17. **Mellado, E., et al.** 2005. Targeted gene disruption of the 14-α-sterol demethylase (*cyp51A*) in *Aspergillus fumigatus* and its role in azole drug susceptibility. Antimicrob. Agents Chemother. **49:**2536–2538.
18. **Mellado, E., et al.** 2007. A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. Antimicrob. Agents Chemother. **51:**1897–1904.
19. **Mortensen, K. L., et al.** 2010. Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain Antimicrob. Agents Chemother. **54:**4545–4549.
20. **Nascimento, A. M., et al.** 2003. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. Antimicrob. Agents Chemother. **47:**1719–1726.
21. **Pfaller, M. A., et al.** 2008. In vitro survey of triazole cross-resistance among more than 700 clinical isolates of *Aspergillus* species. J. Clin. Microbiol. **46:**2568–2572.
22. **Pfaller, M. A., et al.** 2009. Wild-type MIC distribution and epidemiological cutoff values for *Aspergillus fumigatus* and three triazoles as determined by the Clinical and Laboratory Standards Institute broth microdilution methods. J. Clin. Microbiol. **47:**3142–3146.
23. **Rodriguez-Tudela, J. L., et al.** 2008. Epidemiological cutoffs and cross-resistance to azole drugs in *Aspergillus fumigatus*. Antimicrob. Agents Chemother. **52:**2468–2472.
24. **Slaven, J. W., et al.** 2002. Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, *atrF*, in the presence of itraconazole in an itraconazole-resistant clinical isolate. Fungal Genet. Biol. **36:**199–206.
25. **Snelders, E., et al.** 2008. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. PLoS. Med. **5:**e219.
26. **Turnidge, J., G. Kahlmeter, and G. Kronvall.** 2006. Statistical characterization of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. Clin. Microbiol. Infect. **12:**418–425.
27. **Turnidge, J., and D. L. Paterson.** 2007. Setting and revising antibacterial susceptibility breakpoints. Clin. Microbiol. Rev. **20:**391–408.
28. **van der Linden, J. W. M., et al.** 2009. Azole-resistant central nervous system aspergillosis. Clin. Infect. Dis. **48:**1111–1113.
29. **Verweij, P. E., E. Mellado, and W. J. G. Melchers.** 2007. Multiple-triazole-resistant aspergillosis. N. Engl. J. Med. **356:**1481–1483.
30. **Verweij, P. E., S. J. Howard, W. J. G. Melchers, and D. W. Denning.** 2009. Azole-resistance in *Aspergillus*: proposed nomenclature and breakpoints. Drug Resist. Updat. **12:**141–147.
31. **Verweij, P. E., E. Snelders, G. H. J. Kema, E. Mellado, and W. J. G. Melchers.** 2009. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? Lancet Infect. Dis. **9:**789–795.
32. **Walsh, T. J., et al.** 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin. Infect. Dis. **46:**327–360.